Biophysical Studies and Intracellular Destabilization of pH-sensitive Liposomes

Françoise Van Bambeke[a,*], Anne Kerkhofs[a], André Schanck[b], Claude Remacle[c], Etienne Sonveaux[d], Paul M. Tulkens[a], and Marie-Paule Mingeot-Leclercq[a]

[a]Unité de Pharmacologie Cellulaire et Moléculaire, Brussels; [b]Unité de Chimie Physique Moléculaire et de Cristallographie, Louvain-La-Neuve; [c]Laboratoire de Biologie Cellulaire, Unité de Biologie Animale, Louvain-La-Neuve; and [d]Unité de Chimie Pharmaceutique et de Radiopharmacie, Brussels; Université Catholique de Louvain, Belgium

ABSTRACT: We examined changes in membrane properties upon acidification of dioleoylphosphatidylethanolamine/cholesterylhemisuccinate liposomes and evaluated their potential to deliver entrapped tracers in cultured macrophages. Membrane permeability was determined by the release of entrapped calcein or hydroxypyrene-1,3,6-trisulfonic acid (HPTS)-*p*-xylene-bis-pyridinium bromide (DPX); membrane fusion, by measuring the change in size of the liposomes and the dequenching of octadecylrhodamine-B fluorescence; and change in lipid organization, by ^{31}P nuclear magnetic resonance spectroscopy. Measurement of cell-associated fluorescence and confocal microscopy examination were made on cells incubated with liposomes loaded with HPTS or HPTS-DPX. The biophysical studies showed (i) a lipid reorganization from bilayer to hexagonal phase progressing from pH 8.0 to 5.0, (ii) a membrane permeabilization for pH <6.5, (iii) an increase in the mean diameter of liposomes for pH <6.0, and (iv) a mixing of liposome membranes for pH <5.7. The cellular studies showed (i) an uptake of the liposomes that were brought from pH 7.5–7.0 to 6.5–6.0 and (ii) a release of ~15% of the endocytosed marker associated with its partial release from the vesicles (diffuse localization). We conclude that the permeabilization and fusion of pH-sensitive liposomes occur as a consequence of a progressive lipid reorganization upon acidification. These changes may develop intracellularly after phagocytosis and allow for the release of the liposome content in endosomes associated with a redistribution in the cytosol.

Paper no. L8278 in *Lipids 35,* 213–223 (February 2000).

Delivery of macromolecules and other nonpermeant constituents into the cytosol of target cells is essential for the development of many new therapeutic approaches. Conventional liposomes are often ineffective in this context since they tend to bring their load to lysosomes (1). pH-sensitive liposomes (2) may represent a significant improvement for intracellular drug unloading thanks to their progressive, acid-driven destabilization along the endocytic pathway (3). These liposomes, indeed, not only are susceptible to releasing their content in acid vacuoles, such as late endosomes and lysosomes, but also are capable of promoting the transfer of encapsulated tracers to the cytosol (4). Accordingly, they have been assessed in a series of studies for potential therapeutic applications, including antitumor and antibacterial chemotherapy (5–6), and for the delivery of proteins, oligonucleotides, or even DNA (7–9).

In the present study, we examined a typical type of pH-sensitive liposomes made of dioleoylphosphatidylethanolamine and an anionic component, cholesterylhemisuccinate (DOPE/CHEMS). The small cross-sectional area of the headgroup of phosphatidylethanolamine, relative to that of the acyl chains, confers to this molecule a propensity to spontaneously form an inverted hexagonal phase H_{II} structure, a particular organization involved in membrane destabilization and fusion (10). At alkaline or neutral pH, however, the negatively charged CHEMS will reduce the intermolecular repulsions of phosphatidylethanolamine head groups and stabilize the structure in a bilayer organization. Yet, upon acidification, CHEMS will become less charged and will no longer hinder the reorganization of phosphatidylethanolamine in an H_{II} structure, ensuring the destabilization of the membrane structure (3). This mechanism has been documented concerning the increase in membrane permeability (11–16) and fusogenicity (2,12,14,17) of liposomes, and to a lesser extent, the cellular delivery of the vesicles' contents (1,4,18,19). Yet the changes in polymorphic organization of the lipids (20) remain largely undefined in this context. Moreover, most of these studies have used different types and compositions of liposomes, which make their conclusions difficult to generalize because lipid composition critically determines the pH sensitivity of acid-labile liposomes (4,11). In the present study, we have therefore undertaken to characterize, in a more systematic fashion, the biophysical phenomena (including changes in polymorphic organization) leading to an effective *in vitro* release of membrane-impermeant probes from one type of liposomes of a fixed composition (DOPE/CHEMS, 7:3 molar ratio), and have examined whether such release occurs in cells. DOPE/CHEMS liposomes were systematically com-

*To whom correspondence should be addressed at UCL 73.70 avenue E. Mounier 73, 1200 Brussels; Belgium. E-mail: vanbambeke@facm.ucl.ac.be

Abbreviations: CHEMS, cholesterylhemisuccinate; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, *p*-xylene-bis-pyridinium bromide; HPTS, hydroxypyrene-1,3,6-trisulfonic acid; LUV, large unilamellar vesicles; MLV, large multilamellar vesicles; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; R18, octadecylrhodamine B; RPMI, Roswell Park Memorial Institute.

pared to vesicles made of dioleoylphosphatidylcholine and CHEMS (DOPC/CHEMS), since phosphatidylcholine does not spontaneously form an H_{II} structure and is therefore non-fusogenic.

MATERIALS AND METHODS

Liposome preparation. Large unilamellar vesicles (LUV) were used throughout all experiments, but large multilamellar vesicles (MLV) were also included for ^{31}P nuclear magnetic resonance (NMR) studies. Both DOPE/CHEMS and DOPC/CHEMS liposomes were made at a phospholipid/CHEMS molar ratio of 7:3. Depending on the experiments and on the sensitivity of the method used, the volume of buffer varied between 1 and 5 mL, and the concentration of lipids, between 1 and 50 mg/mL. MLV were obtained by hydration of the dry lipid films for 1 h under nitrogen at 37°C in a 40 mM glycine-NaOH solution adjusted to pH 11 and containing the fluorescent probes (see below). They were thereafter submitted to five successive cycles of freezing (at −80°C) and thawing (at 37°C). LUV were obtained by extruding the resulting suspension 10 times through two superposed polycarbonate filters (pore size, 100 nm; Nucleopore Costar Corporation, Badhoevedorp, The Netherlands) under a nitrogen pressure of 17 bars, in a 10-mL Thermobarrel Extruder (Lipex Biomembranes, Vancouver, Canada) (21,22). The actual phospholipid concentration of each preparation was determined by phosphorus assay, as decribed previously (22), and adjusted to the desired value by dilution in the appropriate buffer just prior to each experiment. (All concentrations of liposomes are expressed by reference to their total lipid concentration, based on phospholipid determination and on a molar ratio of phospholipid/CHEMS of 7:3. In preliminary experiments, we checked that the phospholipid/cholesterol molar ratio was effectively very close to 7:3 after the hydration and extrusion procedures.) Liposomes were stored under nitrogen at 4°C until the beginning of the experiment, to minimize the risk of extensive lipid hydrolysis and oxidation (23), and were used within 24 h. The lack of chemical hydrolysis of phosphatidylethanolamine in our experimental conditions was checked by preparing DOPE/CHEMS liposomes with a trace amount of phosphatidylethanolamine 1-palmitoyl-2-(1-^{14}C)-linoleoyl (100 μCi/mmol of DOPE), and letting them age at 4°C for 24 h. Samples were then analyzed by thin-layer chromatography using $CHCl_3/CH_3OH/NH_3$ (25%)/H_2O 24:16:2:1 (by vol) as mobile phase. The radioactivity associated with free fatty acid and lysophosphatidylethanolamine was then measured and found to be less than 2% of the total amount of radioactivity recovered, whereas approximately 94% co-migrated with phosphatidylethanolamine.

Studies on liposomes. (i) Permeability studies. The release of calcein [entrapped at a self-quenching concentration (18,22,24)] and of HPTS [hydroxypyrene-1,3,6-trisulfonic acid (25)] coentrapped with and quenched by DPX (*p*-xylene-bis-pyridinium bromide (11)] from the liposomes was followed by the increase of fluorescence upon dilution following their leakage from the vesicles. Calcein was prepared in 6 N NaOH and purified by chromatography using a Sephadex® LH-20 column, as described previously (22). The final solution had a concentration of 74 mM calcein, a pH of 11, and an osmolarity of 448 mOsm/kg (measured by the freezing point technique; model 3C2, Advanced Cryomatic Osmometer, Advanced Instruments, Needham Heights, MA). HPTS and DPX were dissolved in a 40 mM glycine-NaOH mixture adjusted to pH 11 (393 mOsm/kg) at a concentration of 31.8 and 35 mM, respectively (4,11). In preliminary experiments, we checked that DPX effectively quenched the fluorescence of HPTS for this concentration ratio. These solutions were then used for hydration of the lipid films as described above. After liposome preparation at a lipid concentration of 30 mM, the unencapsulated dye was removed by minicolumn centrifugation (22). Before each experiment, liposomes were adjusted to a total lipid concentration of 7.14 μM and 2.16 μM for calcein- and HPTS-release studies, respectively, using isoosmotic phosphate buffers of pH ranging from 8.0 to 5.0. Calcein fluorescence was measured at room temperature on a PerkinElmer 2000 Fluorescence Spectrophotometer [PerkinElmer, Beaconsfield, United Kingdom; λ_{exc}, 490 nm; λ_{em}, 516 nm (22)]; the inner effect was checked and found to be negligible. The fluorescence of free calcein in solution was found to be time- and pH-dependent, and our measurements were therefore systematically corrected for by appropriate factors. These factors were calculated by measuring the fluorescence of calcein solutions at all the pH values investigated as a function of the time and by comparing these values to that a calcein solution incubated for the same period of time and at pH 7.5. Fluorescence of HPTS was recorded at room temperature, using a λ_{em} of 520 nm and λ_{exc} of 390 and 450 nm (25), using a LS-30 Fluorescence Spectrophotometer (PerkinElmer). The percentage of dye release at any given time was defined as the ratio between the fluorescence measured at that time and that observed at the same time after disruption of the liposomes by exposure to 0.1% Triton X-100 in the same buffer. For each pH- and time-condition tested, the fluorescence values recorded were corrected for the influence of Triton X-100 by comparing the fluorescence value measured for a calcein solution in the absence of Triton to that measured in the presence of the detergent. A leakage efficiency factor, defined as the ratio between the percentage of marker released at a given pH to that measured at pH 7.5, was then used to assess the lability of the liposomes upon pH change.

(ii) Mixing of the liposome membranes: studies with octadecylrhodamine B (R18). Mixing of the liposome membranes was followed by measuring the fluorescence increase of R18, a lipid-soluble probe, upon dilution in the membrane occurring by fusion between labeled and unlabeled liposomes (26,27). Labeled liposomes were obtained by incorporating R18 in the dry lipid film at a concentration of 5.7% of the total lipids. Labeled and unlabeled liposomes, prepared at 2 mM, were mixed at a ratio of 1:4 and diluted to a final concentration of 5 μM by addition of appropriate buffers at the time of the experiment (40 mM cacodylate buffers from pH 7.4 to 5.0 and 40 mM acetate buffer for pH 4.5). Fluorescence was recorded at room temperature (λ_{exc}, 560 nm; λ_{em}, 590 nm; the

inner filter effect was checked and found to be negligible) on an LS-30 PerkinElmer fluorimeter. The percentage of fusion was calculated by comparing the fluorescence values observed after liposome mixing to that measured for 25 μM liposomes labeled by 1.16% of R18 (i.e., an identical total amount of marker diluted five times in membranes).

(iii) Size studies. The apparent size of the liposomes diluted to 50 μM in 40 mM cacodylate buffers of pH ranging from 8.0 to 5.0 was measured by quasi-elastic light scattering spectroscopy (28) using a Coulter Nano-sizer N4 MD particle Analyzer (Coulter Electronics Inc., Luton, England) at an angle of 90°, using both unimodal and size distribution analysis modes to determine the mean diameter and the full size distribution profile of each preparation, respectively.

(iv) Polymorphic behavior of the lipids as determined by ^{31}P NMR. The phase behavior of liposomes was determined as a function of pH and temperature. Classically, three types of phases can be distinguished on the basis of their ^{31}P NMR signals. The so-called bilayer signal is characterized by a high-field maximum and a low-field shoulder; the inverted hexagonal phase, by an inverse symmetry and a twofold reduced width; and the isotropic signal, by a sharp symmetric signal. In practice, MLV give a bilayer signal due to their organization in concentric bilayers, where rapid motion of phospholipid molecules along their long axis results in axial symmetry and partially averages the chemical shift anistropy (29). LUV give an isotropic signal, because the ^{31}P atoms are submitted to rapid isotropic motion owing to the rapid tumbling of the vesicles (30). As stated in the introduction, phosphatidylethanolamine has a propensity to organize itself in hexagonal phase (especially upon warming), in which the molecules project radially from the center of a cylinder of very small radius. The rapid rotation of the cylinders causes further averaging of chemical shift anistropy, explaining the reversed shape and the reduced width of the spectrum by comparison with the bilayer spectrum (29). To compare the appearance of this hexagonal phase starting from an isotropic or a bilayer phase, we performed NMR studies on both LUV and MLV. Liposome suspensions prepared at an initial lipid concentration of 75 mM were diluted in the appropriate buffers (Na carbonate from pH 10.0 to 9.2; Tris-HCl from pH 9.0 to 7.0; citric acid-NaOH from pH 6.2 to 5.2; and Na citrate-HCl from pH 5.0–3.0) and mixed with 0.2 mL D_2O (for locking on the deuterium signal) in 10 mm RMN tubes (final lipid concentration, 12.5 mM). The actual pH value of each suspension was measured with a minielectrode and a 691 Metrohm pH meter (Herisau, Switzerland), at both 30 and 70°C. All spectra were obtained at 101.3 MHz with a WM 250 Bruker instrument. Typical Fourier transform conditions were as follows: spectral width 20 kHz; 8 K data acquisition points; flip angle 60° (17 μs); 1 s repetition time. Five thousand free induction decays (FID) were accumulated with broad-band proton powergated decoupling. A line broadening of 50 Hz was applied to the FID before Fourier transformation. Measurements were done as a function of temperature in the range 30–70°C in the increasing or decreasing mode. After each change of temperature, the sample was allowed to equilibrate during 30 min before accumulation. The lipid phase proportions were calculated as described earlier (31).

Studies on cultured cells. (i) Cell culture and incubation with liposomes. J774 macrophages, a continuous reticulosarcoma cell line of murine origin (32), were grown as monolayers in 5% CO_2/95% air, at 37°C in an RPMI (Rosewell Park Memorial Institute) 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 1.45% (wt/vol) $NaHCO_3$, and antibiotics (streptomycin 50 μg/mL, penicillin 50 IU/mL). Confluent cells were suspended by incubation with trypsin (0.5%)/EDTA (0.2%) in phosphate-buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4; pH 7.4), centrifuged, and plated in six-well plates for 3 d in RPMI medium supplemented with fetal calf serum, $NaHCO_3$, and antibiotics, as decribed above. At the time of the experiment, the cells were washed three times with PBS and reincubated during 15 min with 2 mL PBS containing liposomes (90 μM total lipid concentration) loaded with HPTS or HPTS-DPX. In preliminary experiments, the total fluorescence of the liposome preparations was determined after disruption by exposure to Triton X-100 (0.1%) and corrected for Triton X-100 influence as described above. These studies showed that the amount of HPTS or HPTS/DPX entrapped in DOPC/CHEMS was slightly larger than that entrapped in DOPE/CHEMS liposomes (30% in excess). After 15 min of uptake at 37°C, the cells were washed 10 times with ice-cold PBS and returned in growth medium at 37°C for different chase times.

(ii) Fluorescence assay. The cells were detached by trypsinization, pelleted by centrifugation, washed twice with ice-cold PBS, and gently resuspended in PBS. Viability and membrane integrity were checked by measuring the release of the cytosolic enzyme lactate dehydrogenase. HPTS fluorescence was recorded at a λ_{em} of 520 nm upon excitation at 363, 390, 450, or 488 nm and the signal expressed by reference to the protein cell content (33). The wavelengths of 390 and 450 nm correspond to the maximum in the excitation spectrum of HPTS at acidic and neutral pH, respectively (25), whereas those at 363 and 488 nm were the λ_{exc} used for confocal microscopy studies (see below). The 450/390 fluorescence ratio of HPTS was then used to evaluate the pH at which the probe was exposed in cells, based on calibration curves constructed by preparing the probe in phosphate buffers of known pH (between 5 and 8).

(iii) Confocal microscopy. Cells were treated as described above, except that they were cultivated in Lab-Tek Permanox culture chambers (Nunc, Roskilde, Denmark) allowing for the observation of living cells at high magnification under an inverted microscope. Images were recorded during incubation at 37°C using a Bio-Rad MRC 1024-UV laser scanning confocal microscope (Hemel Hempstead, United Kingdom) operated under control of the Lasersharp 2.10 software. Conditions of imaging were: filters E2 and UBHS, 63× oil-immersion objective, zoom 3, PMT2 (photomultiplier) and PMT3, iris = 3.0, gain = 1300–1500, black level = 0, laser power 0.3–3%. The first channel (PMT2) was set to collect the fluorescence signal

emitted upon excitation at 488 nm, and this signal was treated to appear in green on the screen and micrographs. The second channel (PMT3) was set to read the fluorescence emitted upon excitation at 363 nm and this signal appears in red on the screen and micrographs. Although the excitation wavelengths, which were imposed by the equipment, are not optimal, they nevertheless allowed for a clear-cut analysis of the pH differences in HPTS environment. The pH at which the fluorescence signals recorded upon excitation at 363 and 488 nm are equal was determined to be ~7.15, setting therefore the value for shifting the appearance of the tracer from green to red.

Materials. DOPE and DOPC were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), and radiolabeled phosphatidylethanolamine (specific radioactivity = 54 mCi/mmol), from Amersham International plc (Amersham, United Kingdom). Calcein and CHEMS were purchased from Sigma Chemical Co (St. Louis, MO). HPTS, DPX, and R18 came from Molecular Probes Inc. (Eugene, OR). Cell culture media and fetal calf serum were obtained from Gibco-Biocult (Peisley, Scotland). Other products were of analytical grade and obtained from E. Merck (Darmstadt, Germany).

RESULTS

Studies with liposomes. (i) Permeability studies. Figure 1A shows the release of calcein from LUV as a function of the time of incubation in buffers of different pH. DOPE/CHEMS liposomes released a large proportion (~ 60%) of calcein almost instantaneously when exposed to pH 5.0, with no further release after this first burst. The effect was highly dependent on pH since less than 10% release was observed at pH 7.5. DOPE/CHEMS liposomes exposed to pH 5.5 showed an intermediate behavior. In contrast, DOPC/CHEMS liposomes released a much lower proportion of calcein at pH 5.0, and this release proceeded slowly over the first 10 min of observation (Fig. 1B). Figure 1C shows the comparative release of calcein at 5 min over pH values from 8.0 to 5.0. The data confirm that acidity exerts a marked influence on calcein-release from DOPE/CHEMS liposomes, with a significant effect already observed at pH 6.0 ($P < 0.05$ by t-test). In contrast, DOPC/CHEMS liposomes appear considerably less sensitive to the decrease of pH. In parallel experiments, we measured the release of another marker (HPTS/DPX) from DOPE/CHEMS liposomes (Fig. 1C) with essentially similar results. HPTS/DPX did not significantly leak out from DOPC/CHEMS liposomes under the same conditions (data not shown).

(ii) Liposome membranes mixing studies. Figure 2A shows the fluorescence of R18 upon mixing of labeled and unlabeled LUV made of DOPE/CHEMS at different pH values. Liposomes exposed to pH 5.0 quickly (<5 min) showed a marked signal increase which amounted to approximately 10 times that recorded for the same liposomes exposed to pH 7.4. Liposomes exposed to pH 5.4 showed an intermediate behavior. (More time seems to be needed to reach a plateau value at pH 5 than at pH 5.4; because no measurement was possible for time periods shorter than 30 s, we assume that the dequenching proceeds very quickly during these first 30 s, and more

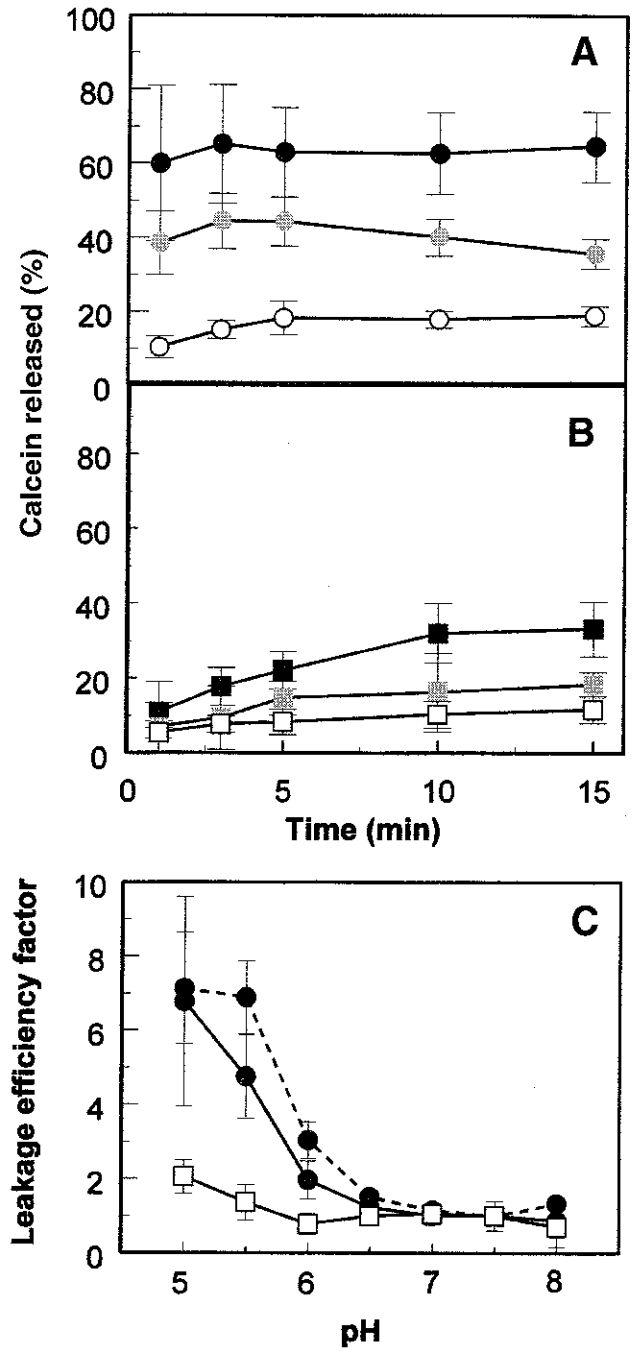

FIG. 1. Release of fluorescent tracers from large unilammelar vesicles (LUV) upon acidification. (A) Percentage of calcein released from dioleoyl phosphatidylethanolamine/cholesterylhemisuccinate (DOPE/CHEMS) or (B) dioleoylphosphatidylcholine/CHEMS (DOPC/CHEMS) liposomes after dilution and incubation at 37°C in isoosmotic phosphate buffers (closed symbols, pH 5.0; grey symbols, pH 5.5; open symbols, pH 7.5). Data shown are the mean values of three independent measurements in single liposome preparations. (C) Leakage efficiency factor (see Materials and Methods section) of DOPE/CHEMS (closed circles) and DOPC/CHEMS (open squares) liposomes loaded with calcein (——) or with hydroxypyrene;1,3,6-trisulfonic acid–*p*-xylene-bis-pyridinium bromide (HPTS-DPX) (----; DOPE/CHEMS only) 5 min after dilution in isoosmotic buffers of different pH. Results are the means ± SD of three independent measures on a single preparation of liposomes.

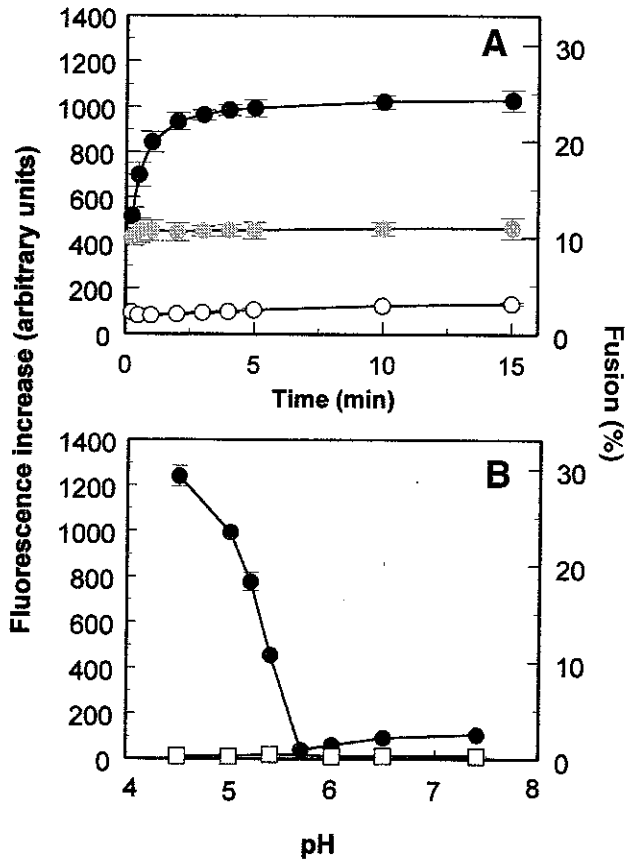

FIG. 2. Octadecylrhodamine B fluorescence dequenching in mixtures of labeled and unlabeled LUV upon acidification. (A) fluorescence recorded for DOPE/CHEMS liposomes after dilution and incubation of the mixed liposome population in 40 mM buffers (closed circles, pH 5.0; grey circles, pH 5.4; open circles, pH 7.4). Each curve is the mean of three independent determinations in single liposome preparations. (B) fluorescence recorded 5 min after dilution in 40 mM buffers for DOPE/CHEMS liposomes (closed circles) and DOPC/CHEMS liposomes (open squares). The right ordinate shows the percentage of fusion. Results are the mean ± SD of three independent measures on a single preparation of liposomes. For abbreviations see Figure 1.

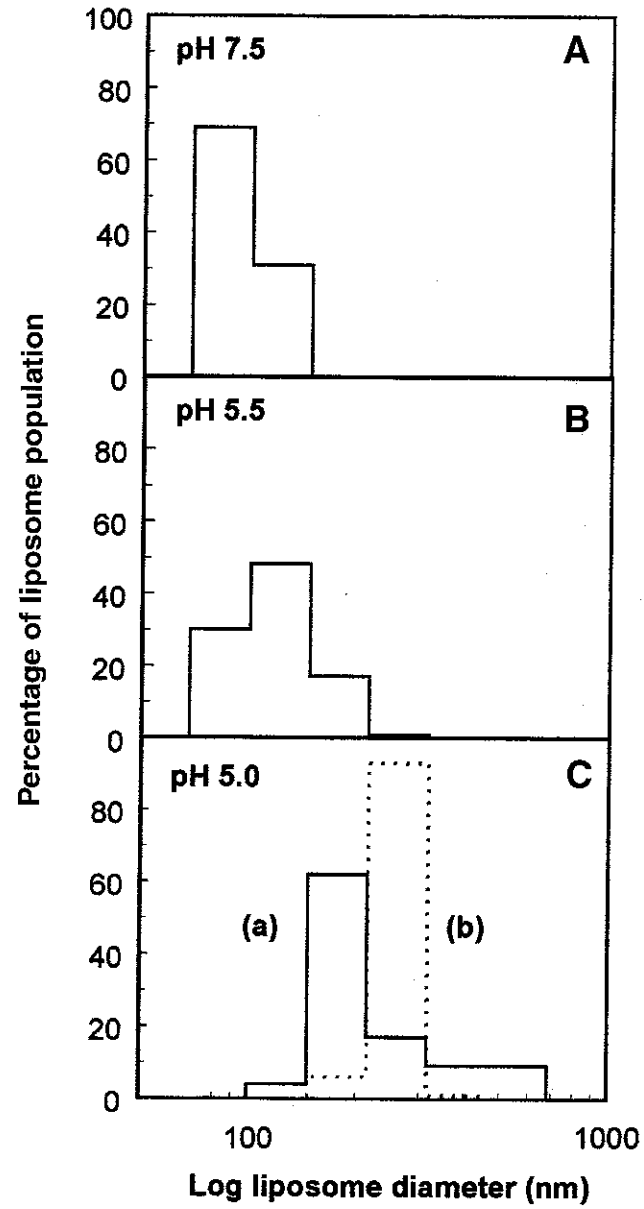

FIG. 3. Size distribution analysis of DOPE/CHEMS LUV determined by light-scattering spectroscopy 5 min after dilution and incubation at 37°C in buffers of different pH. For samples exposed to (C) pH 5.0, one reading was made after acidification [diagram in solid line (a)] and one additional reading was made after titration back to pH 7.5 [diagram in dotted line (b)]. For abbreviations see Figure 1.

slowly thereafter; thus time-dependence of the increase can only be assessed at pH 5.0.) These experiments were then repeated over a whole range of pH values, and DOPE/CHEMS liposomes were compared to DOPC/CHEMS liposomes. Figure 2B shows that the increase of the signal became significant at pH ≤ 5.4. DOPC/CHEMS liposomes did not show significant fluorescence increase throughout the whole range of pH values. The data of Figure 2 were also used to calculate the percentage of liposome fusion and this is shown on the right ordinate. Finally, similar experiments were also run in phosphate buffers (as used in permeability studies) to rule out any influence of the buffer system used. No difference were found when comparing data at the same pH with the phosphate and cacodylate buffer systems.

(iii) Liposome size studies. Unimodal analysis of the particle size showed that the mean diameter of DOPE/CHEMS LUV increased from 120 nm at pH 7.5 to 250 nm at pH 5.0, whereas the mean diameter of DOPC/CHEMS liposomes remained nearly constant at 100 nm in the same range of pH. The size distribution analysis of the DOPE/CHEMS liposomes illustrated in Figure 3 showed a narrowly distributed population at pH 7.5, but a progressive and marked shift toward large-diameter vesicles at acid pH. Titration back to pH 7.5 of liposomes exposed to pH 5.0 did not reverse this effect, and liposomes still presented a larger diameter (mean = 295 nm).

(iv) Polymorphism studies. The polymorphic behavior of DOPE/CHEMS LUV was studied upon temperature and pH

variations using ^{31}P NMR spectroscopy. Figure 4 (left panel) shows the spectra obtained at three different pH values at 30 and 70°C. Quantitative analysis of these spectra and of those obtained at other pH values is shown in the right panel of Figure 4. In alkaline medium and at 30°C, the spectrum was symmetric, with a half-height width of 3 ppm, and it can be considered as that of an isotropic-like phase. A decrease of pH caused the appearance of a spectrum characteristic of a hexagonal phase, showing a low-field peak and a high-field shoulder with a chemical shift anisotropy ($\Delta\sigma$) reduced by a factor of two compared to the $\Delta\sigma$ of a "bilayer" spectrum (10,29). Warming of the liposomes to 70°C caused a narrowing of the isotropic signal in alkaline medium and increased the proportion of hexagonal phase at intermediate pH values, but had no significant effect on the spectrum shapes of hexagonal phase at low pH values. By using the spectra obtained at 30°C for pH 3.7 and 9.3 as references for hexagonal and isotropic phases, respectively, the quantitative analysis showed that the lipidic phase reorganization occurring upon acidification started, at 30°C, from a pH close to 8.0 but became prominent at pH ~ 5.6. Below this value, most of the lipids appeared to be organized in hexagonal phase. At 70°C, the spectra at pH 4 and 9.3 were taken as references for hexagonal and isotropic phases. The curves were very similar but slightly (approximately 0.4 pH units) displaced to less acidic values.

Similar studies were performed on MLV to better evidence the phase transitions. As shown in Figure 5 (left panel), these liposomes displayed a typical bilayer spectrum at 30°C and alkaline pH, with a high-field peak and a low-field shoulder. In contrast, the mixture was clearly organized in hexagonal phase at acidic pH. The phase transformation upon acidification was progressive and accompanied by the appearance of a low proportion of isotropic-type phase detected by a small peak at 0 ppm at pH 7.8. After warming to 70°C, the isotropic signal became detectable at pH 8.7 and was predominant at pH 7.8. After recooling to 30°C, the spectrum observed at pH

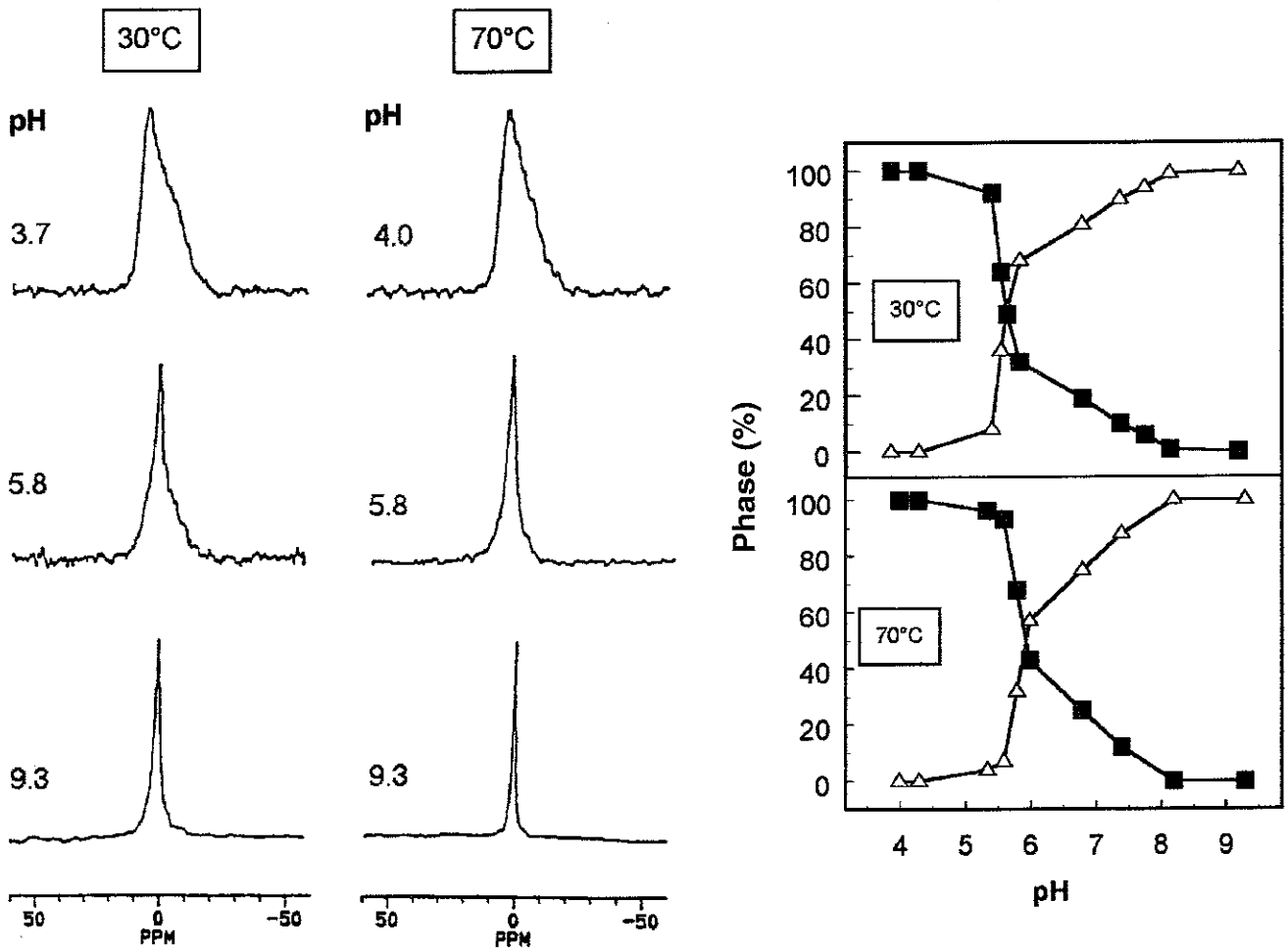

FIG. 4. ^{31}P nuclear magnetic resonance (NMR) spectroscopy of DOPE/CHEMS LUV. Left panel: typical spectra obtained at three different pH values at two temperatures. Right panel: phase behavior of the vesicles over the whole range of pH values investigated at both temperatures. Quantitative assignment to hexagonal (closed squares) or isotropic (open triangles) phases was made for data obtained at 30°C, by reference to the spectra obtained at pH 3.7 (hexagonal phase) or 9.3 (isotropic phase), respectively, and for data obtained at 70°C, by reference to the spectra obtained at pH 4.0 (hexagonal phase) or 9.3 (isotropic phase), as described in Reference 31. Data shown are the mean of successive determinations obtained with SD ~5%. For abbreviations see Figure 1.

7.3 showed a prominent symmetrical and narrow signal with a half-height width ($\Delta\nu_{1/2}$) of 4 ppm. We then quantified these changes using the spectra at pH 8.7 and 30°C, at pH 7.3 and 30°C after recooling (30°C final), and at pH 3.7 and 30°C as reference to a bilayer phase, an isotropic phase, and an hexagonal phase, respectively. The results, presented in Figure 5 (right panel) show that 50% of the phospholipids adopt a hexagonal phase organization at a pH close to neutrality. This transformation from bilayer to hexagonal phase involved a passage through isotropic structures, the proportion of which drastically increased after one cycle of heating and cooling.

Studies with cultured cells. (i) Fate of entrapped HPTS. Figure 6 (upper panel) shows the accumulation and cellular fate of HPTS encapsulated in DOPE/CHEMS and DOPC/CHEMS LUV, in J774 macrophages during a first exposure to the vesicles for a 15-min period (uptake) followed by a 45-min washout (chase; total incubation time: 60 min). Panel A shows that HPTS, whether detected by excitation at 450 or 390 nm, rapidly accumulates in cells exposed to DOPE/CHEMS liposomes. During the chase period, the signal obtained by excitation at 450 nm declined while that obtained by excitation at 390 nm remained stable with a trend toward an increase. HPTS encapsulated in DOPC/CHEMS liposomes was accumulated to a considerably lesser extent, but its fate during the chase was qualitatively similar to that of HPTS encapsulated in DOPE/CHEMS liposomes. To get more insight on the intracellular fate of HPTS, we present in the panel B the ratio of the signals recorded upon excitation at 450 and 390 nm. This shows that a steady and significant shift of pH occurs in the environment of the probe, both with DOPE/CHEMS and DOPC/CHEMS liposomes from early uptake (pH 7.0–7.5) to late chase phase (pH 6.0–6.5); some

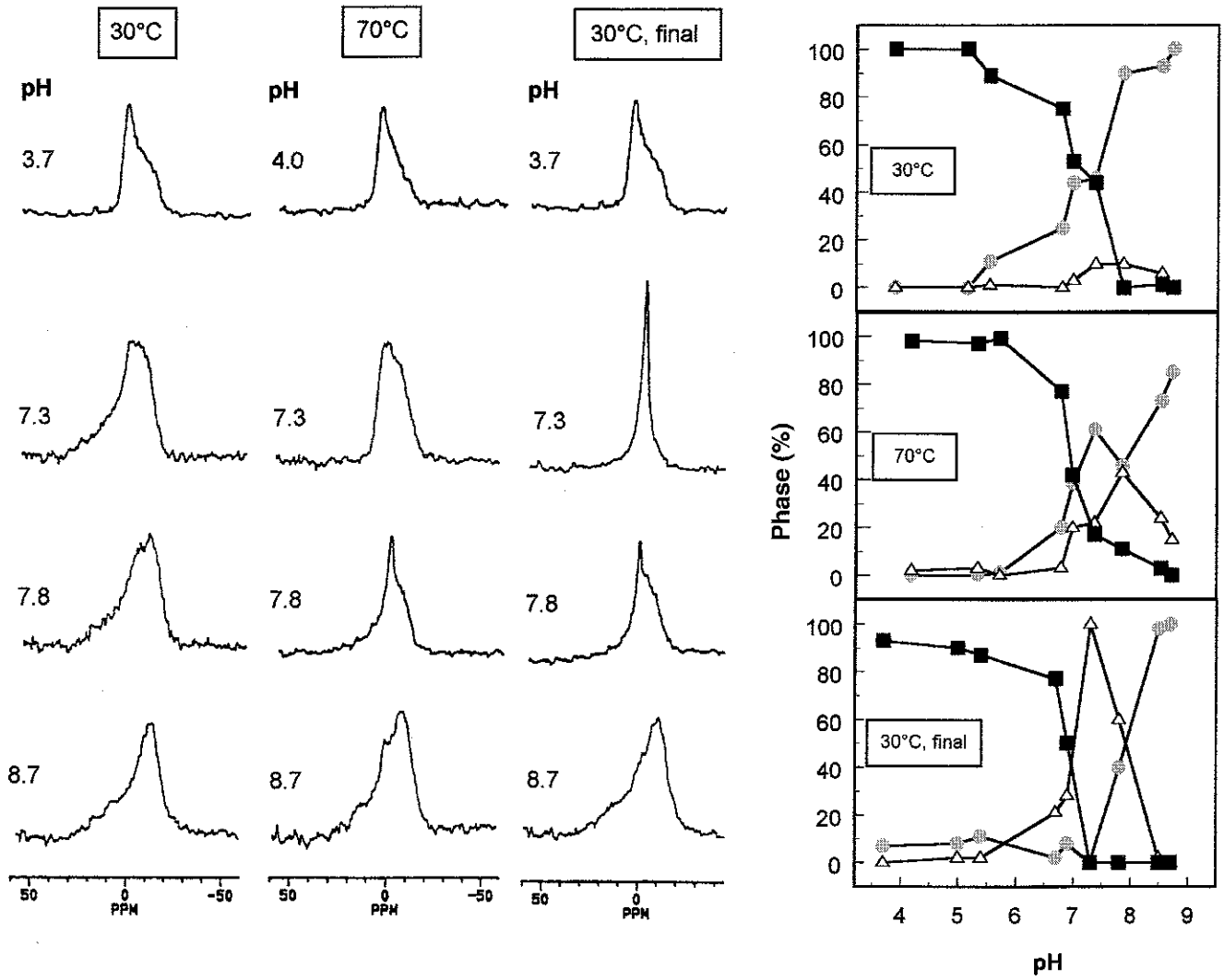

FIG. 5. ^{31}P NMR spectroscopy of DOPE/CHEMS large multilamellar vesicles (MLV). Left panel: typical spectra obtained at four different pH values at three temperatures. Right panel: phase behavior of the vesicles over the whole range of pH values investigated at the three temperatures. Quantitative assignment to hexagonal (closed squares), bilayer (grey circles), or isotropic (open triangles) phases was made by reference to the spectra obtained at 30°C and pH 3.7 (hexagonal phase), pH 8.7 (bilayer phase), or 30°C final and pH 7.3 (isotropic phase), respectively, as described in Reference 31. Data shown are the mean of successive determinations obtained with SD ~5%. For abbreviations see Figures 1 and 4.

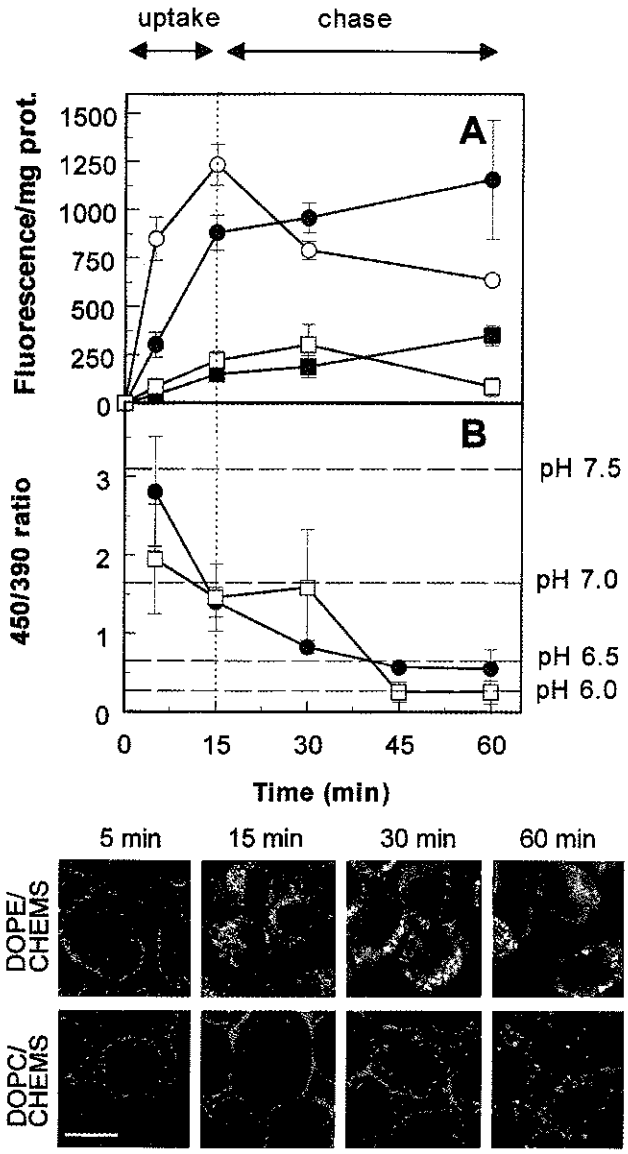

FIG. 6. Accumulation and distribution of fluorescence in J774 macrophages during incubation in the presence of HPTS-containing LUV (uptake) and subsequent transfer to liposome-free medium (chase). Circles, DOPE/CHEMS vesicles; squares, DOPC/CHEMS vesicles. (A) Readings obtained with suspensions of living cells upon excitation at 450 nm (open symbols) or 390 nm (closed symbols); (B), ratio of the recordings made upon excitation at these two wavelengths. Results are the mean of three independent experiments (±SD) (these ratios were used to calculate the mean pH to which HPTS is exposed, and the corresponding values are shown on the right ordinate). Bottom panel: confocal microscopy of cells treated as indicated in the upper panels. Cells were illuminated at 488 nm (green look-up table) and 363 nm (red look-up table). The yellow color results from the colocalization of green and red signals. Bar = 10 μm. For abbreviations see Figure 1.

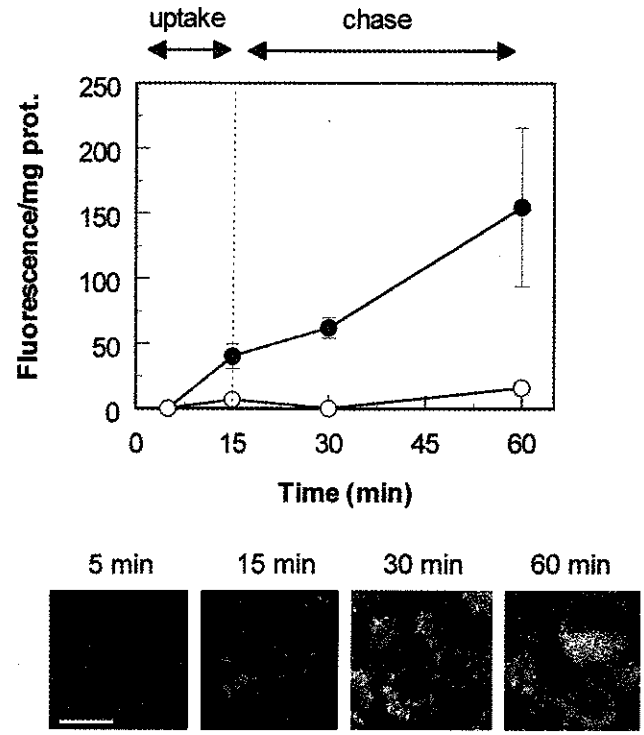

FIG 7. Accumulation and distribution of fluorescence in J774 macrophages incubated in the presence of DOPE/CHEMS LUV containing HPTS together with DPX. The protocol and the experimental conditions were similar to those of Figure 6. Upper panel: readings obtained with cell suspensions (open symbols, λ_{exc} 450 nm; closed symbols, λ_{exc} 390 nm). Lower panel: confocal microscopy observations. Bar = 10 μm. For abbreviations see Figure 1.

delay, however, was noted for DOPC/CHEMS liposomes (to make the link with the confocal microscopy studies, all measurements were also made at 488 and 363 nm, with essentially similar results). The confocal microscopic studies analysis (illustrated in the lower panel of Fig. 6) first confirmed that the accumulation of HPTS in cells exposed to DOPE/CHEMS liposomes was considerably larger than that of the same probe entrapped in DOPC/CHEMS vesicles. With both types of liposomes, the tracer initially appeared as tiny dots located at the periphery of the cell (set to a green color). With DOPE/CHEMS liposomes, these structures became progressively enlarged and more centrally located in a perinuclear fashion, while also gradually emitting a larger proportion of the signal in the red channel, the yellow color resulting from the colocalization of green and red signals. Some of these structures were already visible at 5 min. After 45 min chase, most of the tracer was detected as large red/yellow patches, often with a diffuse appearance. With DOPC/CHEMS liposomes, the tracer still displayed a peripheral appearance after 30 min of incubation. It eventually, but slowly, became associated with larger structures, some of which appeared as red/yellow dots. Yet it was never present in a diffuse fashion.

(ii) Release of HPTS. Figure 7 (upper panel) shows that suspensions of cells incubated with DOPE/CHEMS LUV loaded with HPTS and DPX display almost no signal when excited at 450 nm. At the end of the uptake period, however, they showed a minor signal when excited at 390 nm, the value of which increased throughout the chase period. In confocal microscopy (lower panel), only a few red structures were seen at the end of the uptake period. During the chase, increasingly larger yellow/red patches, with a diffuse appearance, were observed, sometimes surrounded by tiny green spots. No signal

was detected when macrophages were incubated with DOPC/CHEMS liposomes loaded by HPTS and DPX (results not shown).

DISCUSSION

pH-sensitive liposomes have been developed for the purpose of enhancing the cytosolic delivery of drugs and other entrapped solutes through destabilization in the acid milieu of the endosomal/phagosomal apparatus (2–4). We selected DOPE/CHEMS liposomes since vesicles of this composition show a greater stability than liposomes made of DOPE and oleic acid (34), and also because CHEMS induces destabilization through transition to inverted hexagonal (H_{II}) phase (20) in a physiological pH range [7 to 5 (35)]. DOPC/CHEMS liposomes were used as controls since these vesicles cannot undergo reorganization to H_{II} phase, which is considered essential for membrane destabilization and fusion (10). As structural modifications could be the initiating factor of the destabilization of pH-sensitive liposomes upon acidification, we have focused our attention on the changes in lipid organization as well as their relation with membrane permeabilization and fusion.

A first observation in this respect is that change in polymorphic organization of the lipids occurs over a wide pH range and that the hexagonal-phase proportion increases from pH 8.0 down to ~6.0. At this pH value, it affects a large proportion of the lipids and probably reaches a critical threshold to cause alterations of other membrane properties. This threshold cannot, however, be more quantitatively defined in LUV. The broad component seen in the ^{31}P NMR spectra at acid pH, which is typical of a hexagonal phase (10), may indeed include a symmetrical broader component associated with fused liposomes of a still sufficiently small size (see below) to undergo motional averaging of $\Delta\sigma$ (30). The role of structures giving rise to an isotropic signal in the process of phase transition is further substantiated by the ^{31}P NMR studies made on MLV. The highly mobile structures responsible for the isotropic signal may also be involved in membrane fusion (36). However, a quantitative relationship between the proportion of these structures in the membrane and the fusion process cannot be established since MLV are too heterogeneous in size to be used in fusion assays. In addition, LUV, which were therefore used for fusion studies, reorganize in hexagonal phase at a lower pH than MLV. The endothermic character of the transition clearly appears for LUV, since it occurs at a higher pH at 70 than at 30°C. This effect of temperature is particularly dramatic for MLV, since a rise from 30 to 70°C increases the percentage of isotropic-type structures from ~10 to ~40%, and cooling down to 30°C causes a complete transformation of the spectrum in a narrow symmetrical signal. It therefore appears that heating and cooling induce the formation of isotropic-type structures in an irreversible manner. The nature of this structural change is difficult to interpret on the basis of ^{31}P NMR results alone and further investigations, out of the main scope of this paper, are probably needed to independently determine the influences of the temperature and the duration of the cycle on membrane behavior. Whatever the exact nature of these changes, the main conclusion is that both LUV and MLV undergo changes in lipid organization upon acidification susceptible to alter critical membrane properties such as permeability and fusion capacity.

A second observation in this study is that DOPE/CHEMS liposomes require more membrane structure reorganization for fusion than for permeabilization, since there is a difference of approximately 0.5 pH units between the onset of each phenomenon (see data on calcein release on the one side and light-scattering spectroscopy and R18 dequenching on the other side). Our data therefore support and extend those obtained by Collins *et al.* (17), who also reported that leakage occurs at a less acidic pH than membrane fusion. Quite surprisingly, we found that a 100% leakage or fusion could not be achieved, at least in the range of pH investigated, suggesting that a part of the liposome population is resistant to acidification or that membrane perturbations are not sufficient to cause the complete release of the entrapped probe or the fusion of all the vesicles. Incomplete release of a tracer entrapped in DOPE/CHEMS liposomes containing higher proportions of CHEMS was also noted by other investigators (11). In the present study, the kinetic data unambiguously show that a plateau has been reached. It has been proposed (11) that destabilization of pH-sensitive liposomes is primarily mediated by bilayer contact. The degree of permeabilization may therefore be directly related to the lipid concentration. However, in these studies, the authors (11) did not specifically consider whether the aggregation process is followed by fusion of the liposomes. The mixing of lipid components we detected is actually highly suggestive of a true fusion process rather than a simple aggregation, since the increase in LUV size was moderate (only 2–3 times the diameter of control liposomes) and irreversible, and since R18 fluorescence increased very rapidly upon liposome mixing. The present data are therefore very similar to those we reported earlier for negatively charged liposomes incubated with typical fusogenic agents like melittin or (β-diethylaminoethylether)hexestrol (these agents cause a two to five times increase in liposome diameter and an immediate dequenching of R18 fluorescence). They are also in sharp contrast with what we found for aggregating agents like spermine and gentamicin, which induce an at least 10-fold increase in liposome diameter associated with a slow increase of R18 fluorescence (26,37). Altogether, the biophysical studies discussed so far strongly suggest that DOPE/CHEMS liposomes of the composition we selected may become destabilized and release their contents when pH falls from >7 to approximately 6. This range corresponds to that encountered along the endocytic pathway (38). The results of the cellular studies with HPTS-entrapped liposomes (in which the tracer is used to quantify the liposome uptake and to estimate the pH at which it becomes exposed) concur with this consideration. By assuming a rapid equilibration of protons through the liposome membrane (25), it clearly appears that HPTS entrapped in

DOPE/CHEMS or DOPC/CHEMS vesicles travels through compartments whose pH decreases progressively from 7.4 to ~6. It must, however, be stressed that the pH values obtained by the examination of cell suspensions (Fig. 6, upper panel) represent an average value for a material that, as evidenced by confocal microscopy (Fig. 6, lower panel), is spread among vacuoles whose pH values range from neutrality to a low value. Our studies also show that cells handle DOPE/CHEMS and DOPC/CHEMS liposomes very differently. First, the fluorescence signal recorded in cells was markedly lower for DOPC/CHEMS liposomes than for DOPE/CHEMS liposomes. This cannot be attributed to a difference in the amount of probe entrapped (see Materials and Methods section) and must therefore be ascribed to a low endocytic rate of DOPC/CHEMS liposomes, as already observed in P388D1 macrophages (4). This clearly points to the importance of liposome composition for uptake, beyond a simple variation in charge (39). Second, the confocal microscopy studies show that HPTS entrapped in DOPC/CHEMS liposomes remains for about 30 min in the periphery of the cell and at neutral pH before being transferred to vesicles of lower pH. Because our calibration studies indicate that the shift from the green to the red signal should occur around pH 7.15, the data therefore suggest that DOPC/CHEMS liposomes remain associated with the cell surface, or with invaginations of the plasma membrane, for quite a time before being transferred to early endosomes and other acidic vacuoles. In contrast, HPTS entrapped in DOPE/CHEMS liposomes appears to move more quickly into the cell where it progressively shows a diffuse appearance in parallel with a marked shift from green to yellow and red. Again, taking into account the pH at which this shift is observed (~7.15), we interpret these images as indicating a rapid internalization into early endosomes followed by a partial release of the tracer in the cytosol, the pH of which appears to be precisely around this value in J774 macrophages (40). An effective release of the content of DOPE/CHEMS liposomes into the cytosol also is largely evidenced by the results of the experiments using vesicles containing both HPTS and its quenching agent DPX. Comparing the values of the fluorescence signal obtained with these liposomes (Fig. 7) to that obtained with vesicles without the quenching agent (Fig. 6), we suggest that approximately 15% of the HPTS has been made free within 45 min after endocytosis, a figure close to that found by Chu *et al.* (4) for DOPE/CHEMS (3:2) liposomes. This value may, however, be underestimated, since a significant increase in HPTS fluorescence will be only observed upon high dilution of the dye/quencher mixture (41).

In conclusion, our data confirm the potential usefulness of DOPE/CHEMS liposomes as pH-sensitive vehicles for the intracytosolic delivery of an entrapped tracer. This delivery seems to occur without grossly affecting cell viability, suggesting interesting applications for therapeutics. Our biological observations are supported by biophysical data pointing to fruitful strategies for further improving the design and construction of this type of liposomes.

ACKNOWLEDGMENTS

F.V.B. and M.P.-M.L. are respectively Chargé de Recherches and Chercheur Qualifié of the Belgian Fonds National de la Recherche Scientifique. Francine Renoird, Marie-Claire Cambier, and Christelle Flore provided expert technical assistance. This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale (grants nos. 9.4541.95F, 3.4516.94 and 9.451492), the Fonds National de la Recherche Scientifique (grant no. 9.4546.94), the Actions de Recherches Concertées 94/99-172 of the Direction Générale de la Recherche Scientifique—Communauté Française de Belgique, Belgium.

REFERENCES

1. Straubinger, R.M., Hong, K., Friend, D., and Papahadjopoulos, D. (1983) Endocytosis of Liposomes and Intracellular Fate of Encapsulated Molecules: Encounter with a Low pH Compartment After Internalization in Coated Vesicles, *Cell 32,* 1069–1079.
2. Connor, J., Yatvin, M.B., and Huang, L. (1984) pH-Sensitive Liposomes: Acid-Induced Liposome Fusion, *Proc. Natl. Acad. Sci. USA 81,* 1715–1718.
3. Chu, C.J., and Szoka, F.C. (1994) pH-Sensitive Liposomes, *J. Liposome Res. 4,* 361–395.
4. Chu, C.J., Dijkstra, J., Lai, M.Z., Hong, K., and Szoka, F.C. (1990) Efficiency of Cytoplasmic Delivery by pH-Sensitive Liposomes to Cells in Culture, *Pharm. Res. 7,* 824–834.
5. Connor, J., and Huang, L. (1986) pH-Sensitive Immunoliposomes as an Efficient and Target-Specific Carrier for Antitumor Drugs, *Cancer Res. 46,* 3431–3435.
6. Lutwyche, P., Cordeiro, C., Wiserman, D.J., St. Louis, M., Uh, M., Hope, M.J., and Finlay, B.B. (1998) Intracellular Delivery and Intracellular Activity of Gentamicin Encapsulated in pH-Sensitive Liposomes, *Antimicrob. Agents Chemother. 42,* 2511–2520.
7. Couvreur, P., Fattal, E., Malvy, C., and Dubernet, C. (1997) pH-Sensitive Liposomes: an Intelligent System for the Delivery of Antisense Oligonucleotides, *J. Liposome Res. 7,* 1–18.
8. Nair, S., Zhou, F., Reddy, R., Huang, L., and Rouse, B.T. (1992) Soluble Proteins Delivered to Dendritic Cells *via* pH-Sensitive Liposomes Induce Primary Cytotoxic T Lymphocyte Responses *in vitro, J. Exp. Med. 175,* 609–612.
9. Wang, C.Y., and Huang, L. (1987) Plasmid DNA Adsorbed to pH-Sensitive Liposomes Efficiently Transforms the Target Cells, *Biochem. Biophys. Res. Commun. 147,* 980–985.
10. Seddon, J.M. (1990) Structure of the Inverted Hexagonal (H_{II}) Phase, and Nonlamellar Phase Transitions of Lipids, *Biochim. Biophys. Acta 1031,* 1–69.
11. Ellens, H., Bentz, J., and Szoka, F.C. (1984) pH-Induced Destabilization of Phosphatidylethanolamine-Containing Liposomes: Role of Bilayer Contact, *Biochemistry 23,* 1532–1538.
12. Düzgünes, N., Straubinger, R.M., Baldwin, P.A., Friend, D.S., and Papahadjopoulos, D. (1985) Proton-Induced Fusion of Oleic-Phosphatidylethanolamine Liposomes, *Biochemistry 24,* 3091–3098.
13. Liu, D., and Huang, L. (1989) Small, but Not Large, Unilamellar Liposomes Composed of Dioleoylphosphatidylethanolamine and Oleic Acid Can Be Stabilized by Human Plasma, *Biochemistry 28,* 7700–7707.
14. Collins, D., Litzinger, D.C., and Huang, L. (1990) Structural and Functional Comparisons of pH-Sensitive Liposomes Composed of Phosphatidylethanolamine and Three Different Diacylsuccinylglycerols, *Biochim. Biophys. Acta 1025,* 234–242.

15. Hazemoto, N., Harada, M., Suzuki, S., Kaiho, F., Haga, M., and Kato, Y. (1993) Effect of Phosphatidylcholine and Cholesterol on pH-Sensitive Liposomes, *Chem. Pharm. Bull. 41,* 1003–1006.
16. Tari, A.M., Fuller, N., Boni, L.T., Collins, D., Rand, P., and Huang, L. (1994) Interactions of Liposome Bilayers Composed of 1,2-Diacyl-3-succinylglycerol with Protons and Divalent Cations, *Biochim. Biophys. Acta 1192,* 253–262.
17. Collins, D., Maxfield, F., and Huang, L. (1989) Immunoliposomes with Different Acid Sensitivities as Probes for the Cellular Endocytic Pathway, *Biochim. Biophys. Acta 987,* 47–55.
18. Straubinger, R.M., Düzgünes, N., and Papahadjopoulos, D. (1985) pH-Sensitive Liposomes Mediate Cytoplasmic Delivery of Encapsulated Macromolecules, *FEBS Lett. 179,* 148–154.
19. Kono, K., Igawa, T., and Takagishi, T. (1997) Cytoplasmic Delivery of Calcein Mediated by Liposomes Modified with a pH-Sensitive Poly(ethylene Glycol) Derivative, *Biochim. Biophys. Acta 1325,* 143–154.
20. Lai, M.Z., Vail, W.J., and Szoka, F.C. (1985) Acid- and Calcium-Induced Structural Changes in Phosphatidylethanolamine Membranes Stabilized by Cholesterol Hemisuccinate, *Biochemistry 24,* 1654–1661.
21. Hope, M.J., Bally, M.B., Webb, G., and Cullis, P.R. (1985) Production of Large Unilamellar Vesicles by a Rapid Extrusion Procedure. Characterization of Size Distribution, Trapped Volume and Ability to Maintain a Membrane Potential, *Biochim. Biophys. Acta 812,* 55–65.
22. Van Bambeke, F., Mingeot-Leclercq, M.P., Schanck, A., Brasseur, R., and Tulkens, P.M. (1993) Alterations in Membrane Permeability Induced by Aminoglycoside Antibiotics: Studies on Liposomes and Cultured Cells, *Eur. J. Pharmacol. 247,* 155–168.
23. Grit, M., Zuidam, N.J., Underberg, W.J.M., and Crommelin, D.J.A. (1993) Hydrolysis of Partially Saturated Egg Phosphatidylcholine in Aqueous Liposome Dispersions and the Effect of Cholesterol Incorporation on Hydrolysis Kinetics, *J. Pharm. Pharmacol. 45,* 490–495.
24. Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R., and Hagins, W.A., (1977) Liposome–Cell Interaction: Transfer and Intracellular Release of a Trapped Fluorescent Marker, *Science 195,* 489–491.
25. Straubinger, R.M., Papahadjopoulos, D., and Hong, K. (1990) Endocytosis and Intracellular Fate of Liposomes Using Pyranine as a Probe, *Biochemistry 29,* 4929–4939.
26. Van Bambeke, F., Tulkens, P.M., Brasseur, R., and Mingeot-Leclercq, M.P. (1995) Aminoglycoside Antibiotics Induce Aggregation but Not Fusion of Negatively-Charged Liposomes, *Eur. J. Pharmacol. 289,* 321–333.
27. Hoekstra, D., de Boer, T., Klappe, K., and Wilschut, J. (1984) Fluorescence Method for Measuring the Kinetics of Fusion Between Biological Membranes, *Biochemistry 23,* 5675–5681.
28. Mazer, N.A., Carey, M.C., Kwasnick, R.F., and Benedek, G.B. (1979) Quasi-elastic Light Scattering Studies of Aqueous Biliary Lipid Systems. Size, Shape and Thermodynamics of Bile Salt Micelles, *Biochemistry 18,* 3064–3075.
29. Seelig, J. (1978) ^{31}P Nuclear Magnetic Resonance and the Head Group Structure of Phospholipids in Membranes, *Biochim. Biophys. Acta 515,* 105–140.
30. Burnell, E.E., Cullis, P.R., and De Kruijff, B. (1980) Effects of Tumbling and Lateral Diffusion on Phosphatidylcholine Model Membrane ^{31}P-NMR Lineshapes, *Biochim. Biophys. Acta 603,* 63–69.
31. Schanck, A. (1992) A Method for Determining the Proportions of Different Phases in Hydrated Phospholipids by ^{31}P Nuclear Magnetic Resonance (NMR) Spectroscopy, *Appl. Spectrosc. 46,* 1435–1437.
32. Snyderman, R., Pike, M.C., Fischer, D.G., and Koren, H.S. (1977) Biologic and Biochemical Activities of Continuous Macrophage Cell Lines P 338 D1 and J 774 1, *J. Immunol. 119,* 2060–2066.
33. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193,* 265–275.
34. Straubinger, R.M. (1993) pH-Sensitive Liposomes for Delivery of Macromolecules into Cytoplasm of Cultured Cells, *Methods Enzymol. 194,* 28–36.
35. Ellens, H., Bentz, J., and Szoka, F.C. (1985) H^+ and Ca^{2+} Fusion and Destabilization of Liposomes, *Biochemistry 24,* 3099–3106.
36. Van Bambeke, F., Mingeot-Leclercq, M.P., Brasseur, R., Tulkens, P.M., and Schanck, A. (1996) Aminoglycoside Antibiotics Prevent the Formation of Non-Bilayer Structures in Negatively-Charged Membranes. Comparative Studies Using Fusogenic [bis-(beta-diethylaminoethylether)hexestrol] and Aggregating (spermine) Agents, *Chem. Phys. Lipids 79,* 123–135.
37. Mingeot-Leclercq, M.P., Schanck, A., Ronveaux-Dupal, M.F., Deleers, M., Brasseur, R., Ruysschaert, J.M., Laurent, G., and Tulkens, P.M. (1989) Ultrastructural, Physico-Chemical and Conformational Study of the Interactions of Gentamicin and bis(beta-diethylaminoethylether)hexestrol with Negatively-Charged Phospholipid Bilayers, *Biochem. Pharmacol. 38,* 729–741.
38. Hubbard, A.L. (1989) Endocytosis, *Curr. Opin. Cell Biol. 1,* 675–683.
39. Miller, C.R., Bondurant, B., McLean, S.D., McGovern, K.A., and O'Brien, D.F. (1998) Liposome–Cell Interactions *in vitro*: Effect of Liposome Surface Charge on the Binding and Endocytosis of Conventional and Sterically Stabilized Liposomes, *Biochemistry 37,* 12875–12883.
40. Gan, B.S., Krump, E., Shrode, L.D., and Grinstein, S. (1998) Loading Pyranine *via* Purinergic Receptors or Hypotonic Stress for Measurement of Cytosolic pH by Imaging, *Am. J. Physiol. Cell Physiol. 44,* C1158–C1166.
41. Daleke, D.L., Hong, K., and Papahadjopoulos, D. (1990) Endocytosis of Liposomes by Macrophages: Binding, Acidification and Leakage of Liposomes Monitored by a New Fluorescence Assay, *Biochim. Biophys. Acta 1024,* 352–366.

[Received June 9, 1999, and in revised form November 18, 1999; revision accepted January 4, 2000]